Symbiosis, 27 (1999) 17–32
Balaban, Philadelphia/Rehovot

Stable Transformation of T1 and T2 Transgenic Alfalfa with Antisense-Lectin Constructs

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Received May 1, 1999; Accepted August 1, 1999

Abstract

Antisense constructs of *MsLEC1* and *MsLEC2* (two of the three lectin genes found in alfalfa) have been introduced by *Agrobacterium*-mediated transformation into alfalfa cv. Regen. The resulting MsLEC1AS and MsLEC2AS primary transgenic lines were kanamycin-resistant and contained DNA that hybridized to *nptII*. In addition, Southern analysis demonstrated that some of the lectin gene-hybridizing bands were the same molecular weight as bands hybridizing to the *nptII* probe, indicating intact integration of the transgenes. Following self-pollination, we observed that pod and seed production, as well as viability of seeds from the selfed plants, were lower for the antisense lectin-expressing plants than for the controls. Seedlings derived from selfed antisense transgenic lines were also resistant to kanamycin, indicating that the transgenes were heritable. Moreover, the T2 seedlings exhibited a number of severe developmental abnormalities that had been previously observed in T1 plantlets of comparable developmental age. These results indicate that T2 antisense alfalfa lines are stably transformed and furthermore, that *MsLEC1* and *MsLEC2* are important for the early stages of alfalfa development.

Keywords: Lectins, alfalfa, antisense constructs, seedling development

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Presented at the 8th Congress of the African Association for Biological Nitrogen Fixation, November 23–27, 1998, Cape Town, South Africa

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1. Introduction

Lectins are carbohydrate-binding proteins of non-immune origin that have been extensively characterized from a vast number of organisms (Pusztai, 1991). Numerous functions, such as cell recognition, endocytosis, cell division and differentiation, lymphocyte migration and adhesion, have been ascribed to lectins (Sharon and Lis, 1990; Lasky, 1992; Weis and Drickamer, 1996). In plants, some of the best-described lectins are the legume lectins, in part because they are abundant in seeds and also because they have been extensively utilized in biomedical research (Brewin and Kardailsky, 1997). Moreover, some legume lectins have been proposed to function as part of the host-rhizobia recognition system (see reviews by Kijne et al., 1997; Hirsch 1999). The lectin-recognition hypothesis was formulated to explain host specificity between the symbiotic partners (Hamblin and Kent, 1973; Bohlool and Schmidt, 1974; Dazzo and Hubbell, 1975). It was based on the strong correlation between the inoculation specificity of bacteria of the family Rhizobiaceae on their legume hosts and the ability of host-produced lectins to bind to *Rhizobium* cells.

Díaz et al. (1989) and van Rhijn et al. (1998) utilized gain-of-function experiments to study the involvement of lectins in host recognition. Díaz et al. (1989) introduced the pea lectin gene into *Trifolium repens* (white clover), and van Rhijn et al. (1998) transformed *Lotus corniculatus* plants with the soybean lectin gene *Le1*. *Le1* codes for soybean seed lectin (SBL), which is a D-galactose/*N*-acetyl-D-galactosamine-specific lectin. In both sets of experiments, transforming the host with a foreign lectin resulted in an extension of host range such that a rhizobial species that would not normally nodulate white clover, namely, *Rhizobium leguminosarum* bv. *viciae*, or *L. corniculatus*, namely, *Bradyrhizobium japonicum*, could now nodulate that host. When we inoculated the transgenic *L. corniculatus* plants with an Exo- mutant of *B. japonicum* USDA 110, we found that no nodules were formed above background levels. This suggested that an exopolysaccharide component of the rhizobial cell surface binds to SBL (van Rhijn et al., 1998). However, whether or not a rhizobial exopolysaccharide component is the ligand that binds to lectin in all legume-*Rhizobium* interactions is unknown. These experiments demonstrate that lectins mediate one level of host specificity, but the exact mechanism of how they do this is still incompletely understood.

Loss-of-function investigations have also been used to test the involvement of lectin in host recognition. The best known example is the lectinless (*Le1*$^{-}$) mutant of soybean, cv. "Sooty" (Pull et al., 1978). The ability of this SBL-minus genotype to nodulate in response to inoculation with *B. japonicum* has been presented as critical evidence against the lectin-recognition hypothesis. However, soybean has other lectins in addition to SBL, and these may

compensate for the loss of the product of the *Le1* gene in the lectinless mutant. For example, a 45-kD lectin specific for 4-O-methylglucuronic acid has been isolated from soybean roots, but so far a gene for this lectin has not been identified (Rutherford et al., 1986).

In addition to mutation, another way of generating loss-of-function is through antisense technology. The introduction of an antisense lectin construct by *Agrobacterium*-mediated transformation should result in the inactivation of lectin expression. Alfalfa (*Medicago sativa* L.) is one of the few legumes that is readily transformed by *Agrobacterium tumefaciens*, and thus it serves as an excellent model system for antisense experiments. However, alfalfa, like other medics (Bauchrowitz et al., 1992) has three seed lectin genes: *MsLEC1* (Brill et al., 1995), *MsLEC2* (manuscript in preparation), and *MsLEC3* (W.M. Karlowski and A.M. Hirsch, unpublished results). Although one of the alfalfa lectin genes appears to be a pseudogene (W.M. Karlowski and A.M. Hirsch, unpublished results), introduction of an antisense construct could potentially affect the expression of the other two genes. Thus, we needed to utilize antisense constructs that contained DNA specific to each alfalfa lectin gene.

A preliminary characterization of the immature, primary transgenic alfalfa plants expressing antisense *MsLEC1* and *MsLEC2*-lectin genes was reported earlier (Hirsch et al., 1995). Plants expressing the *MsLEC1*-antisense gene (described earlier as γ plants) and those expressing the *MsLEC2*-antisense gene (previously designated as β plants) are now termed MsLEC1AS and MsLEC2AS plants, respectively. In our earlier work, we showed that the MsLEC1AS and MsLEC2AS plants 1) underwent somatic embryogenesis poorly, 2) displayed abnormalities as young plantlets, and 3) accumulated transgene-encoded RNAs (antisense-RNA) (Hirsch et al., 1995).

In this report, stable integration of the transgenes in DNA of mature primary transformant (T1) MsLEC1AS, MsLEC2AS, and vector-only control plants is examined. Continued transgene activity in the T2 generation is also demonstrated. To our knowledge, this is the first report that demonstrates inheritance of an antisensed symbiosis-associated gene in alfalfa. Previous studies have concentrated exclusively on T1 antisense (AS) alfalfas (Hirsch et al., 1995; Schulze et al., 1998; Temple et al., 1998).

2. Materials and Methods

Plant material

Transgenic MsLEC1AS, MsLEC2AS, and control plants were regenerated after *Agrobacterium tumefaciens*-mediated transformation as described (Hirsch et

al., 1995). Of the 52 independent MsLEC1AS and 134 MsLEC2AS primary transgenic lines examined by RNA blot analysis in our earlier work (Hirsch et al., 1995), three plants from each primary transgenic AS line as well as from the vector control line were chosen for further study. The plants were grown to maturity in a greenhouse under natural daylight conditions, and then self-pollinated after flowering (Brill, 1997). Pods were left to mature and harvested 40 days after pollination. The seeds were removed from all pods, and the seed number per pod was recorded. All seeds were examined under a dissecting microscope.

Healthy-looking seeds were later surface-sterilized in 95% ethanol for 1 hour, followed by three rinses in sterile water. The seeds were scarified and planted in Magenta jars (Magenta Corp., Chicago, IL) containing 50 ml of complete Hoagland's 1/4 strength medium plus kanamycin (150 mg/l) and 0.2% Phytagel (Sigma Chemical Co., St. Louis, MO). The Magenta jars were kept in a Conviron growth chamber at a 27/23°C thermoperiod corresponding to a 16h/8h-light/dark cycle. After germination, the seedlings were examined without knowledge of their identity in random order for resistance (continued growth) or sensitivity (bleaching and subsequent death) to kanamycin and for normal or abnormal seedling development. Representative seedlings were photographed.

Restriction digests and Southern hybridization

Leaves were collected from vector control and AS lines that had been grown for 28–30 months in the absence of antibiotic selection. Genomic DNA was isolated from these leaves as well as from leaves of alfalfa cv. Regen SY (Bingham, 1991) using the procedure of Dellaporta et al. (1983). The DNA was digested with *Eco*RI or *Eco*RV or *Hind*III, and the products were separated on 1.0% agarose-TBE gels, and transferred to Nytran Plus membranes according to the manufacturer (Schleicher and Schuell, Inc., Keene, NH, USA). The blots were hybridized with probes specific for either *MsLEC1* or *MsLEC2* (both probes lack internal *Eco*RI, *Eco*RV, and *Hind*III sites; Hirsch et al., 1995). The *MsLEC1*-specific probe is a 420-kb segment of the *MsLEC1* gene and the *MsLEC2*-specific probe is a 400-kb fragment of the *MsLEC2* gene (Hirsch et al., 1995). The blots were also hybridized with the *nptII*/*nos*3'/LB-probe. This probe, which includes sequences of the selectable marker and the left border, is on a *Bam*HI/*Sal*I fragment of the plant transformation vector pART27 (Gleave, 1992). DNA probes were labeled with αP^{32}dCTP (New England Nuclear, Boston, MA) using an oligolabeling kit (Pharmacia Biotechnology, Alameda, CA). The AS-orientation of the construct containing either the *MsLEC1*- or

MsLEC2-transgene was verified with restriction digests and DNA sequencing (Hirsch et al., 1995).

Blots were hybridized in 5X Denhardt's solution, 5X SSPE, 0.5% SDS, salmon sperm DNA (100 μg/ml), and 50% formamide, at 37°C. The blots were washed under high stringency conditions that resulted in gene-specific hybridization (Hirsch et al., 1995). The highest stringency conditions were 65°C, 0.1X SSPE/0.5% SDS for probing with the *MsLEC1*- and *MsLEC2*-specific probes, and 55°C, 0.5X SSPE/0.5% SDS for probing with the *nptII*-probe. Washed blots were exposed to X-ray film at –70°C with intensifying screens.

RNA blots

Vegetative shoot tips, consisting of the shoot apical meristem as well as subtending leaf primordia and some stem, from two independent plants were utilized for the control and MsLEC2AS RNA samples, and from three plants for the MsLEC1AS RNA isolations. Tissue was frozen in liquid nitrogen and stored at –70°C until needed. RNA was isolated, subjected to electrophoresis, blotting, and probing as described (Hirsch et al., 1995). The double-stranded *MsLEC1*-specific DNA probe was labeled as described above. The blots were washed under high stringency conditions and were exposed to X-ray film for 15 days.

3. Results and Discussion

Stable transformation of mature primary transgenic alfalfa plants

In our initial study of alfalfa plantlets developed under antibiotic selection in tissue culture, 44 of 52 independent MsLEC1AS lines and 125 of 134 of MsLEC2AS lines were transgenic, based on the accumulation of antisense-RNAs in Northern blot analysis (described in Hirsch et al., 1995). In this study, we analyzed RNA from tissue derived from mature plants that originated from some of the positive transgenic lines. These plants were grown in the greenhouse in the absence of selection.

Fig. 1 illustrates a Northern blot of RNAs isolated from greenhouse-grown Regen control, vector control, and MsLEC1AS and MsLEC2AS transgenic shoot tips, and probed with the *MsLEC1*-specific probe. An endogenous transcript of ca. 1.3 kb is present in all the lanes. In addition to the endogenous transcript, a smaller (0.7 kb) antisense transcript is observed in two of three MsLEC1AS plant RNAs (lanes MsLEC1AS BB and CC), indicating that the transgene is expressed and that the plants are stably transformed. In addition, RNA blot analysis showed that control and AS lines accumulated *nptII*-RNAs (data not shown).

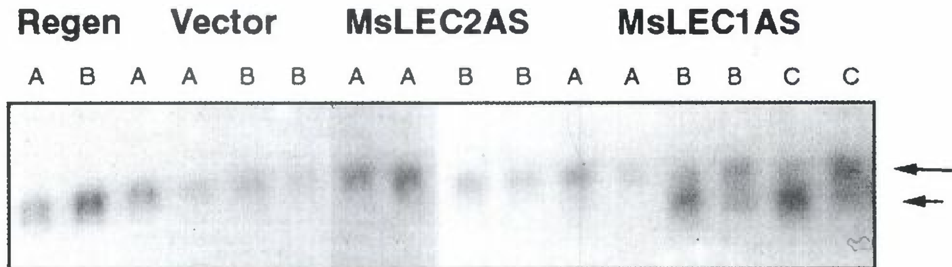

Figure 1. Northern blot analysis of *MsLEC1* endogenous and antisense transcripts in shoot tip RNA isolated from mature plants; 10 µg of RNA were loaded per lane. Lanes 1 and 2 contain RNAs from two different plants (A and B) of the parental, non-transformed cv. Regen and lanes 3–6 contain two samples of RNA from two different (A and B) vector control transgenic plants. Lanes 7–10 contain two samples each of RNA from two different MsLEC2AS plants (A and B). The remaining lanes contain two samples of RNA from three independent MsLEC1AS plants (A, B, and C). The endogenous transcript appears smaller in the Regen lanes, but this is an artifact caused by loading the RNAs near the edge of the gel.

That an endogenous transcript is present in the AS plants is not surprising because introduction of a construct containing antisensed nucleotides does not always result in down-regulation or elimination of the endogenous mRNA. For example, Temple et al. (1993) described a high level of accumulation of an antisense alfalfa GS_1 transcript with no change in the levels of the endogenous GS_1 sense transcript in transgenic tobaccos. If, as they proposed, a stable duplex is formed between the imperfectly matched transcripts, then neither RNA would be degraded (Temple et al., 1993). On the other hand, when Temple et al. (1998) produced transgenic alfalfa plants that contained either of the two antisensed GS_1 genes, there was a reduction in the level of the corresponding glutamine synthetase gene transcripts. However, transcripts corresponding to the antisense gene construct were not detected in any of the alfalfa lines. Moreover, there was no change in GS activity or GS_1 polypeptide levels in the transgenic plants (Temple et al., 1998).

MsLEC2 RNA accumulation in control and AS plants was analyzed using Southern blot hybridization. Genomic DNAs from 10 control, 11 MsLEC1AS, and 11 MsLEC2AS plant lines were analyzed. Representative results from one vector control, two MsLEC1AS, and two MsLEC2AS lines are shown in Fig. 2. Panels A, B, and C were derived from the same Southern blot (except for lanes MsLEC2AS BB in Panel B), and show hybridization of the *MsLEC1*-specific, *MsLEC2*-specific, and *nptII*-probes, respectively. DNA from control and MsLEC2AS lines displayed *MsLEC1*-hybridizing bands of the same sizes when

digested with the same restriction enzymes (Fig. 2A). These bands also matched the band sizes found in the DNA of cv. Regen, from which all the transgenic plants were derived (data not shown). In the control and MsLEC1AS plant DNAs, an *Eco*RI band of about 10 kilobase pairs (kbp) strongly hybridized to the *MsLEC1*-probe, whereas *Eco*RI bands of about 4.0 and 1.7 kbp weakly hybridized and were not visible on all blots. In addition, there was an extra, higher molecular weight *Eco*RI band that hybridized with the *MsLEC1*-probe in the DNA of one of the MsLEC1AS lines; this band also hybridized with the *nptII*-probe (described below). Although there were similarly-sized *Hind*III bands present in DNA from control and MsLEC1AS plants, there were also extra *Hind*III bands that hybridized with the *MsLEC1*-probe in the DNA of the two MsLEC1AS lines (Fig. 2A). The extra bands were not detected in the DNA of control, MsLEC2AS, or Regen plant lines (Figs. 2A and data not shown). The additional *MsLEC1*-hybridizing bands in MsLEC1AS plants represent stably integrated AS-*MsLEC1* transgenes.

When the *MsLEC2*-probe was used, DNAs from control and MsLEC1AS (Fig. 2B), as well as Regen (data not shown) plant lines exhibited similarly-sized hybridizing bands. Moreover, additional *MsLEC2*-hybridizing bands were detected in DNA isolated from the MsLEC2AS lines (Fig. 2B).

To confirm the stable integration of transgenes into control and AS transgenic plants, genomic Southern blots were also probed with *nptII*/*nos* 3'/LB sequences (the *nptII* probe). These sequences are the last of the T-DNA sequences transferred to the plant during transformation with the binary vector pART27 (Gleave, 1992). If the *nptII* sequences are present in the plant genome, all other sequences on the T-DNA should have been co-transferred to the plant genomic DNA due to physical linkage and prior transfer. Control and AS plants all had different patterns of hybridization with the *nptII* probe (Fig. 2C). This result indicates random integration of T-DNA sequences into the genomes of the transgenic alfalfa lines, and confirms stable transformation of these mature plants. Fig. 2C shows a highly variable number of insertion events of the transgene into the genomes of the plant lines. Similar results were obtained with the other AS and control lines that were examined (data not shown). As a negative control, there was no detectable hybridization of the *nptII*-probe to DNA from the non-transformed alfalfa cv. Regen (data not shown). These results also demonstrate that there is efficient alfalfa transformation by the vector pART27, a vector that was previously shown to transform *Nicotiana plumbaginifolia* efficiently (Gleave, 1992).

Portions of both transgenes containing AS- and *nptII*/*nos* 3'/LB sequences would not have been separated by the digestions shown in Fig. 2 (Gleave, 1992; Hirsch et al., 1995). At least one of the additional *MsLEC1*-probe-hybridizing bands in one of the MsLEC1AS plant DNAs was the same molecular weight as a band hybridizing with the *nptII* probe (compare Figs. 2A and 2C, lanes

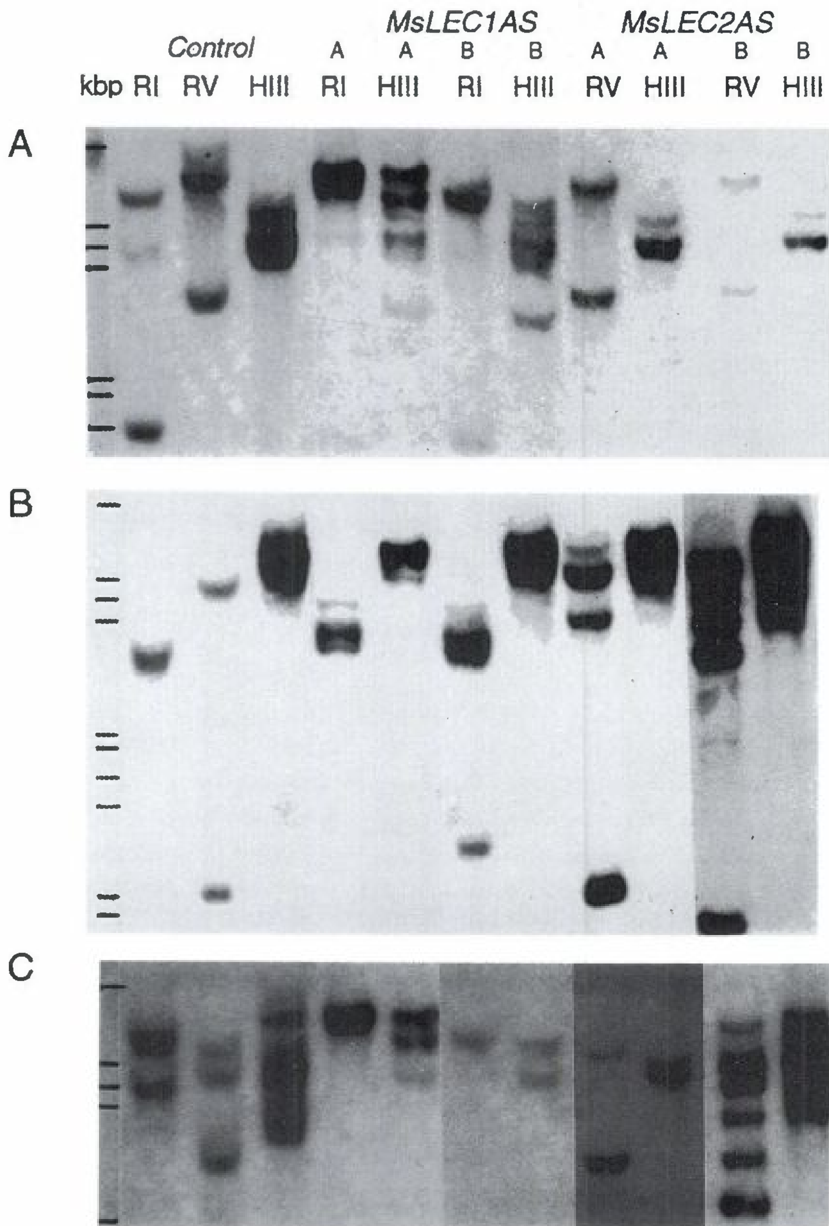

Figure 2. Confirmation of stable transformation of mature transgenic alfalfa using Southern analysis. DNA from transgenic plants was restricted with either *Eco*RI, *Eco*RV, or *Hind*III, subjected to electrophoresis, and transferred to blots for Southern hybridization. The first lane indicates the positions of the molecular weight standards; from top to bottom, the sizes were 21.8, 5.2, 4.2, 3.4, 1.96, 1.90, 1.71, and 1.32 kbp. (For continuation of legend see next page)

MsLEC1AS). This indicates intact integration of at least some of the AS-*MsLEC1* transgenes with the physically linked *nptII/nos* 3'/LB sequences. Similarly, at least one additional *MsLEC2*-hybridizing band in the MsLEC2AS DNAs migrated at the same molecular weight as a band hybridizing with the *nptII* probe (compare Figs. 2B and 2C, lanes MsLEC2AS), indicating intact integration of at least some of the AS-*MsLEC2* transgenes with the physically linked *nptII/nos* 3'/LB sequences.

Stable transformation of T2 alfalfa plants

Several Regen plants and independent lines of the vector control and AS primary transgenic plants were selfed. Even though alfalfa is considered an outcrossing plant, we were successful in generating large numbers of pods for the first selfed generation. The mean number of pods for both MsLEC1AS and MsLEC2AS plants was less than 20% that for the vector controls (data not shown). However, these data were difficult to treat statistically because of differences in plant sizes and potential differences in plant dry weights. On the other hand, Table 1 shows that there was a relatively small, yet statistically significant reduction in the mean seed number per pod in both MsLEC1AS and MsLEC2As lines. A more severe reduction in the mean seed number per pod was observed for the MsLEC1AS pods compared to both sets of controls.

Three types of seeds were evident in the pods of the selfed plants: normal seeds (Fig. 3A), shriveled but viable seeds (Fig. 3B), and aborted seeds (data not shown). The percentage of shriveled but viable seeds was greater for both MsLEC1AS and MsLEC2AS plants compared to the controls (Table 1). The percentage of aborted seeds was nearly twice as high compared to the control for both sets of antisense plants (Table 1).

Figure 2. Continuation. Only (B) shows the lowermost bands of 0.93 and 0.84 kbp. DNA from the control is from a vector-control plant. DNA digests from two different MsLEC1AS (A and B) and two different MsLEC2AS (A and B) plants are presented; 20 µg of DNA were loaded per lane. (A) Probed with the *MsLEC1*-specific probe, washed under high stringency conditions, and exposed to X-ray film for 19 days. (B) The same blot stripped and re-probed with the MsLEC2 probe. The blot was washed under high stringency conditions and exposed to X-ray film for 15 days. (C) The blot was also probed with the *nptII/nos* 3' sequences (Gleave, 1992). Washing was at medium-high stringency and the blot was exposed to X-ray film for six days. This was the first probe used on the blot.

Figure 3. T2 seeds and seedlings. **(A)** Plump seed (ca. 3 mm long) derived from a selfed vector control. **(B)** Shriveled seed (ca. 3 mm long) derived from a selfed AS plant. The darkened areas near the hilum are due to shriveling (sh). **(C)** Regen control seedlings. Arrows point to kanamycin-sensitive leaves, which bleach white. The cotyledons are marked by asterisks. Bar = 1 cm. **(D)** MsLEC1AS seedling. Three cotyledons (*) are evident and some appendages appear to end in roots (arrows). h = hypocotyl. Bar = 5 mm. **(E)** MsLEC2AS seedling. The tip of one of the cotyledons is root-like, and is covered with root hairs (arrow). dsa = deformed shoot apex; lf = leaf; sc = seed coat. Bar = 5 mm. **(F)** An older MsLEC1AS seedling, which appears fairly normal except for its highly branched, negatively gravitropic, root (arrow). The lateral roots are positively gravitropic. Bar = 1 cm.

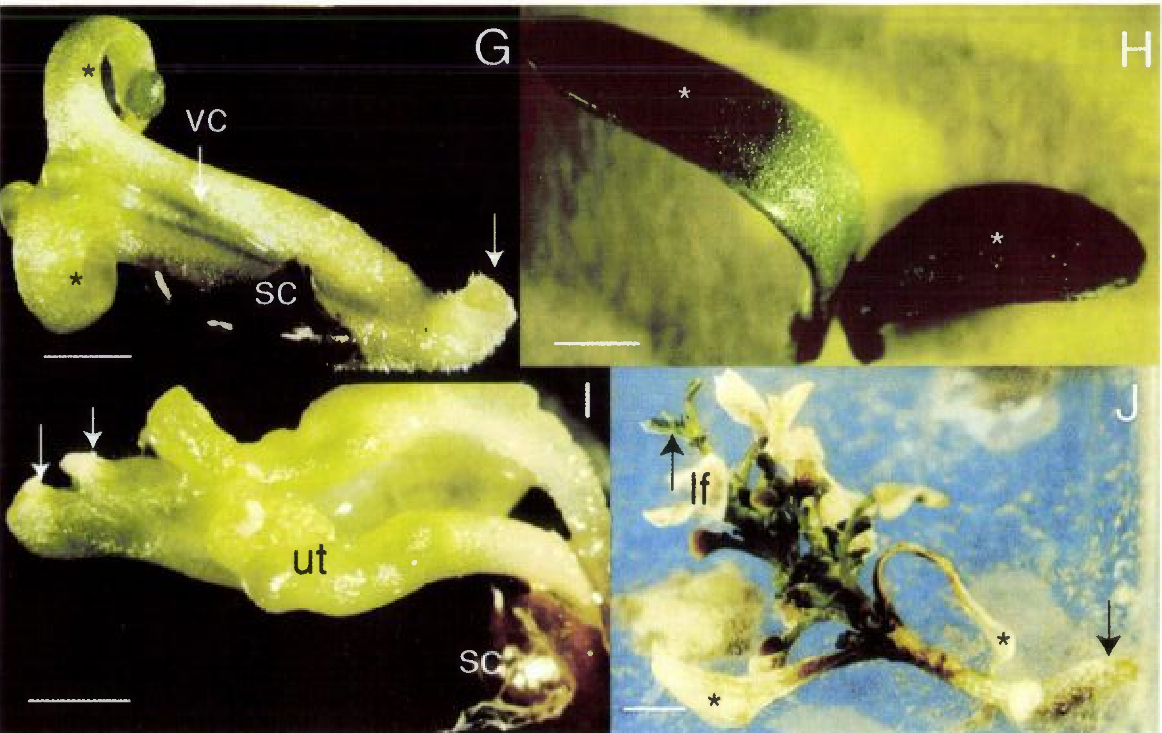

Figure 3. T2 seeds and seedlings. **(G)** Vitreous MsLEC1AS seedling with contorted cotyledons (*). The arrow points to root-like tissue at one end of the seedling. The vascular cylinder (vc) is exposed. sc = seed coat. Bar = 5 mm. **(H)** MsLEC1AS seedling with cotyledons (*) and no other organs. Bar = 5 mm. **(I)** Vitreous MsLEC1AS seedling. Arrows point to tissues that appear root-like. ut = unorganized tissue; sc = seed coat. Bar = 2 mm. **(J)** An 88-day-old MsLEC1AS seedling. Much of the tissue is senescent, including the cotyledons (*). Arrow points to the root. lf = leaf. Bar = 2 mm.

Table 1. Reproduction parameters of control and antisense lectin alfalfa plants

Parameter	Regen controls	Vector controls	MsLEC1AS plants	MsLEC2AS plants
Mean seed no./pod	1.63a* ± 0.81** (431)	1.69a ± 0.90 (1482)	1.27c ± 0.55 (263)	1.45b ± 0.70 (351)
% shriveled seeds	9.20 (674)	7.46 (2439)	20.95 (315)	17.31 (439)
% aborted seeds	6.08 (707)	7.39 (2584)	13.73 (357)	13.57 (516)

*Different letters follow means that differ significantly in analysis of variance (ANOVA) at $P<0.05$. **Standard deviation. The number in parenthesis is the total number examined.

Table 2. Kanamycin response and development of T2 seedlings

Parameter	Regen controls	Vector controls	MsLEC1AS plants	MsLEC2AS plants
Mean % kanamycin -resistance	0a* (88)	74.3b ± 17.5** (232)	91.9c ± 9.7 (130)	88.8c ± 11.7 (168)
Mean % severely abnormal seedlings	1.3a ± 3.7 (88)	3.3a ± 5.4 (232)	21.8b ± 20.2 (130)	19.4b ± 24.9 (168)

*Different letters follow means that differ significantly in analysis of variance (ANOVA) at $P<0.05$. **Standard deviation. The number in parenthesis is the total number examined.

Because the lectin transgenes were linked to *nptII*, which confers kanamycin resistance, we anticipated that the transgenes would be present in the T1 gametes and passed on to the T2 generation. Table 2 and Fig. 3C show that selfed Regen seedlings were sensitive to kanamycin whereas T2 vector control and antisense seedlings were kanamycin-resistant. Surprisingly, significantly fewer vector control seedlings were kanamycin-resistant than either the MsLEC1AS or MsLEC2AS seedlings. We do not know the reason for this result.

The identity of the seedlings was unknown when their development was scored as normal or severely abnormal. Severely abnormal seedlings exhibited one or more of the following symptoms: organ loss or incomplete development, an increase in organ number, aberrant morphology, and for roots, a change in gravitropic response. The mean percentages of severely abnormal MsLEC1AS and MsLEC2AS seedlings were significantly higher than vector control or Regen

seedlings (Table 2). Severely abnormal MsLEC1AS and MsLEC2AS T2 seedlings were always kanamycin-resistant, and in general the abnormalities of MsLEC1AS T2 seedlings tended to be more severe.

These types of abnormalities are shown by the representative seedlings depicted in Fig. 3D–J. For example, Fig. 3D shows a MsLEC1AS T2 seedling with three cotyledons (marked with asterisks) attached to one end of a distorted hypocotyl (h). Also, non-green, root-like appendages that showed negative gravitropism were also present. In the MsLEC2AS seedling illustrated in Fig. 3E, one of the cotyledons appeared to terminate in a root, which was covered with root hairs. In addition, the shoot apex appeared deformed (dsa) although the first trifoliate leaf (lf) looked normal (Fig. 3E). Older MsLEC1AS seedlings with normal leaves also produced roots with aberrant gravitropism; these roots were frequently branched and abnormally thickened (Fig. 3F). The MsLEC1AS seedling shown in Fig. 3G produced twisted cotyledons. It also formed root and shoot apices that failed to divide, and developed what-appears-to-be a partially exposed vascular cylinder (vc; Fig. 3G). Other MsLEC2AS seedlings produced only cotyledons and no other organs (Fig. 3H). Fig. 3I depicts a vitreous MsLEC1AS seedling composed of tissue of uncertain identity and showing abnormal pigmentation. Tissue at the opposite extremities appeared root-like comparable to replacement of cotyledon tips with roots (compare with Fig. 3E). An 88 day-old MsLEC1AS seedling is illustrated in Fig. 3J. The plant is severely stunted, its roots failed to grow, and some leaves failed to expand. In addition, gravitropism was affected, and tissues were prematurely senescent. No connection between the degree of T2 abnormalities and individual T1 parental lines was noted. We did not observe these unusual seedling morphologies in Regen or vector control plant lines.

In spite of efforts to promote their survival, AS seedlings often died, as was the case for the primary transgenic plantlets (Hirsch et al., 1995). Nevertheless, some T2 seedlings survived and like the T1 plants, those that did largely outgrew their seedling abnormalities. DNA from these plants has been amplified by PCR with primers to *nptII*. Preliminary results indicate that a band corresponding to *nptII* is present in the genome of the T2 plants (C.A. Hackworth, E.A. Ayala, and A.M. Hirsch, unpublished results). These T2 plants are currently being examined for their symbiotic phenotype in response to inoculation with wild-type and mutant *Sinorhizobium meliloti*.

In summary, the MsLEC1AS, MsLEC2AS, and vector control alfalfa plants are stably transformed based on molecular and genetic evidence. The MsLEC1AS and MsLEC2AS plant lines will be useful tools for analysis of the numerous effects of expression of antisense-lectin transgenes in alfalfa plants. Some of these effects have been described here and in a previous report (Hirsch et al., 1995). Further description will follow in subsequent publications.

Acknowledgments

This paper was written in partial fulfillment of the Ph.D. degree for LMB to the University of California-Los Angeles. We thank K.L. Wycoff, R.E. Satterthwaite, C.J. Evans, and W.M. Karlowski for help with some of the experiments. C.A. Hackworth and E. A. Ayala are thanked for allowing us to communicate their unpublished results. We also thank members of our lab group, especially W.M. Karlowski, for helpful comments on the manuscript. Support was provided by the USDA-NRICGP 96-35305-3583 to AMH and the UCLA Biotechnology Training Program, which provided fellowship support to LMB.

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